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A process for preparing zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof

Description

The present invention relates to a process for preparing zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof by culturing genetically modified plants which, compared to the wild type, have a reduced ε-cyclase activity caused by double-stranded ε-cyclase ribonucleic acid sequences, to the genetically modified plants and to the use thereof as foodstuffs and feedstuffs and for producing carotenoid extracts.

Carotenoids such as, for example, lycopene, lutein, β-carotene or zeaxanthin, are synthesized de novo in bacteria, algae, fungi and plants. Ketocarotenoids, i.e. carotenoids, containing at least one keto group, such as, for example, astaxanthin, canthaxanthin, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, adonirubin and adonixanthin are natural antioxidants and pigments produced as secondary metabolites by some algae and microorganisms.

Owing to their coloring properties, the carotenoids are used as pigmenting agents and pigmenting aids. Zeaxanthin and lutein, for example, are used in yolk pigmentation, β-carotene serves as an orange pigment in food and beverages, astaxanthin is used as a pigmenting aid in livestock nutrition, especially in trout, salmon and shrimp rearing.

In addition, the carotenoids such as, for example, lutein, zeaxanthin, lycopene, β-carotene and astaxanthin are used in supplementing human and livestock nutrition for the therapy and prevention of diseases, owing to their antioxidant properties.

An economical, biotechnological process for preparing natural carotenoids is of great importance.

WO 00/32788 discloses influencing particular carotenoid ratios in tagetes petals by a combination of overexpression of carotenoid biosynthesis genes and antisense processes.

Although the process disclosed in WO 00/32788 provides genetically modified plants which, compared to the wild type, have an altered carotenoid content, said process has the disadvantage that the content level of carotenoids of the

"β-carotenoid pathway", such as, for example, β-carotene or zeaxanthin, and the purity of said carotenoids, and thus the ratio of carotenoids of the "β-carotenoid pathway", such as, for example β-carotene or zeaxanthin, to the carotenoids of the "α-carotenoid pathway", such as α-carotene or luteine, for example, are not yet satisfactory.

It was therefore the object of the invention to provide an alternative process for preparing zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof by cultivation of plants and, respectively, provide further transgenic plants which produce zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof and which have optimized properties such as, for example, a higher content of zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof in comparison with carotenoids of the "α-carotenoid pathway" and do not have the reported disadvantage of the prior art.

Accordingly, a process for preparing zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof by culturing genetically modified plants which, compared to the wild type, have a reduced ε-cyclase activity caused by double-stranded ε-cyclase ribonucleic acid sequences, was found.

ε-Cyclase activity means the enzyme activity of an ε-cyclase.

ε-Cyclase means a protein which has the enzymic activity of converting a terminal, linear lycopene radical into an ε-ionone ring.

ε-Cyclase, therefore, means in particular a protein which has the enzymic activity of converting lycopene to δ-carotene.

Consequently, ε-cyclase activity means the amount of lycopene converted or the amount of δ-carotene produced in a particular time by the ε-cyclase protein.

Thus, when an ε-cyclase activity is reduced compared with the wild type, the amount of lycopene converted or the amount of δ-carotene produced in a particular time is reduced by the ε-cyclase protein, in comparison with the wild type.

A reduced ε-cyclase activity means, preferably, to partially or essentially completely stop or block, based on different cell-biological mechanisms, the functionality of an ε-cyclase in a

plant cell, plant or part, tissue, organ, cells or seeds derived therefrom.

The ε-cyclase activity in plants may be reduced, compared with the wild type, for example by reducing the amount of ε-cyclase protein or the amount of ε-cyclase mRNA in the plant. Consequently, a reduced ε-cyclase activity, compared with the wild type, may be determined directly or via determining the amount of ε-cyclase protein or the amount of ε-cyclase mRNA of the plant of the invention, in comparison with the wild type.

A reduction in the ε-cyclase activity comprises reducing the amount of an ε-cyclase down to an essentially complete absence of said ε-cyclase (i.e. lack of detectability of ε-cyclase activity or lack of immunological detectability of said ε-cyclase). The ε-cyclase activity (or the amount of ε-cyclase protein or the amount of ε-cyclase mRNA) in the plant, particularly preferably in flowers, is reduced, in comparison with the wild type, preferably by at least 5%, more preferably by at least 20%, more preferably by at least 50%, more preferably by 100%. "Reduction" means in particular also the complete absence of ε-cyclase activity (or of the ε-cyclase protein or ε-cyclase mRNA).

Preference is given to determining the ε-cyclase activity in genetically modified plants of the invention and in wild-type or reference plants under the following conditions:

The ε-cyclase activity may be determined *in vitro* according to Fraser and Sandmann (Biochem. Biophys. Res. Comm. 185(1) (1992) 9-15), when the buffer potassium phosphate (pH 7.6), the substrate lycopene, paprika stromal protein, NADP+, NADPH and ATP are added to a particular amount of plant extract.

Particular preference is given to determining the ε-cyclase activity in genetically modified plants of the invention and in wild-type or reference plants according to Bouvier, d'Harlingue and Camara (Molecular Analysis of carotenoid cyclase inhibition; Arch. Biochem. Biophys. 346(1) (1997) 53-64):

The in-vitro assay is carried out in a volume of 0.25 ml. The reaction mixture comprises 50 mM potassium phosphate (pH 7.6), different amounts of plant extract, 20 nM lycopene, 0.25 mg of paprika chromoplastid stromal protein, 0.2 mM NADP+, 0.2 mM NADPH and 1 mM ATP. NADP/NADPH and ATP are dissolved in 0.01 ml of ethanol with 1 mg of Tween 80 immediately before addition to the incubation medium. After a reaction time of 60 minutes at 30°C,

the reaction is stopped by adding chloroform/methanol (2:1). The reaction products extracted into chloroform are analyzed by means of HPLC.

An alternative assay with radioactive substrate is described in Fraser and Sandmann (Biochem. Biophys. Res. Comm. 185(1) (1992) 9-15). Another analytical method is described in Beyer, Kröncke and Nievelstein (On the mechanism of the lycopene isomerase/cyclase reaction in Narcissus pseudonarcissus L. chromoplast,; J. Biol. Chem. 266(26) (1991) 17072-17078).

Depending on the context, the term "plant" may mean the parent plant (wild type) or a genetically modified plant of the invention or both.

Preferably, and in particular in cases in which the plant or the wild type cannot be classified unambiguously, "wild type" for the reduction in ε-cyclase activity and the increase in the content of zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof means a reference plant.

Said reference plant is Tagetes erecta, Tagetes patula, Tagetes lucida, Tagetes pringlei, Tagetes palmeri, Tagetes minuta or Tagetes campanulata, particularly preferably *Tagetes erecta*.

In the process of the invention, the ε-cyclase activity is reduced by introducing at least one double-stranded ε-cyclase ribonucleic acid sequence, also referred to as ε-cyclase dsRNA hereinbelow, or of an expression cassette or expression cassettes ensuring expression thereof, into plants.

Included are those processes in which said ε-cyclase dsRNA is directed against an ε-cyclase gene (i.e. genomic DNA sequences such as the promoter sequence) or an ε-cyclase transcript (i.e. mRNA sequences).

Genetically modified plants which, in comparison with the wild type, have a reduced ε-cyclase activity caused by double-stranded ε-cyclase ribonucleic acid sequences mean, according to the invention, that the ε-cyclase activity is reduced by using double-stranded ε-cyclase ribonucleic acid sequences. This process of gene regulation by means of double-stranded RNA ("double-stranded RNA interference", also referred to as dsRNA process) is known per se and described, for example, in Matzke MA et al. (2000) Plant Mol Biol 43:401-415; Fire A. et al (1998)

Nature 391:806-811; WO 99/32619; WO 99/53050; WO 00/68374; WO 00/44914; WO 00/44895; WO 00/49035 or WO 00/63364. Express reference is made hereby to the processes and methods described in the citations indicated.

According to the invention, "double-stranded ribonucleic acid sequence" means one or more ribonucleic acid sequences which, either theoretically, owing to complementary sequences, for example according to the base pair rules by Watson and Crick, and/or practically, for example owing to hybridization experiments, are capable of forming double-stranded RNA structures in vitro and/or in vivo.

The skilled worker appreciates that the formation of double-stranded RNA structures represents a state of equilibrium. The ratio of double-stranded molecules to corresponding dissociated forms is preferably at least 1 to 10, preferably 1:1, particularly preferably 5:1, most preferably 10:1.

A double-stranded ε-cyclase ribonucleic acid sequence or else ε-cyclase dsRNA, preferably means an RNA molecule which has a double-stranded structural region and comprises, in said region, a nucleic acid sequence which

a) is identical to at least part of the ε-cyclase transcript intrinsic to said plant and/or

b) is identical to at least part of the ε-cyclase-promoter sequence intrinsic to said plant.

In the process of the invention, therefore, the ε-cyclase activity is preferably reduced by introducing into the plant an RNA which has a double-stranded structural region and comprises, in said region, a nucleic acid sequence which

a) is identical to at least part of the ε-cyclase transcript intrinsic to said plant and/or

b) is identical to at least part of the ε-cyclase-promoter sequence intrinsic to said plant.

The term "ε-cyclase transcript" means the transcribed part of an ε-cyclase gene, which comprises, in addition to the ε-cyclase-encoding sequence, also noncoding sequences such as, for example, also UTRs.

An RNA which "is identical to at least part of the ε-cyclase promoter sequence intrinsic to said plant" preferably means that the RNA sequence is identical to at least part of the theoretical transcript of the ε-cyclase promoter sequence, i.e. the corresponding RNA sequence.

"Part" of the ε-cyclase transcript intrinsic to the plant or of the ε-cyclase promoter sequence intrinsic to the plant means partial sequences which may range from a few base pairs up to complete sequences of the transcript or of the promoter sequence. The optimal length of said partial sequences may be determined readily by the skilled worker by means of routine experiments.

The length of the partial sequences is usually at least 10 bases and no more than 2 kb, preferably at least 25 bases and no more than 1.5 kb, particularly preferably at least 50 bases and no more than 600 bases, very particularly preferably at least 100 bases and no more than 500, most preferably at least 200 bases or at least 300 bases and no more than 400 bases.

The partial sequences are preferably selected so as to achieve a specificity as high as possible and not to reduce activities of other enzymes, whose reduction is not desired. It is therefore advantageous to choose for the ε-cyclase dsRNA partial sequences parts of the ε-cyclase transcript and/or partial sequences of the ε-cyclase promoter sequences which do not occur in other sequences.

In a particularly preferred embodiment, therefore, the ε-cyclase dsRNA comprises a sequence which is identical to part of the ε-cyclase transcript intrinsic to the plant and which comprises the 5′ end or the 3′ end of the nucleic acids coding for a ε-cyclase and intrinsic to the plant. Untranslated regions in the 5′ or 3′ of the transcript are particularly suitable for preparing selective double-stranded structures.

The invention further relates to double-stranded RNA molecules (dsRNA molecules) which cause a decrease in an ε-cyclase when introduced into a plant organism (or a cell, tissue, organ or propagation material derived therefrom).

In this context, a double-stranded RNA molecule for reducing expression of an ε-cyclase (ε-cyclase dsRNA) preferably comprises

a) a sense-RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least part of a sense-RNA ε-cyclase transcript, and

b) an antisense-RNA strand which is essentially, preferably fully, complementary to the RNA sense strand under a).

With respect to the dsRNA molecules, ε-cyclase nucleic acid sequence or the corresponding transcript preferably means the sequence according to SEQ ID No. 4 or a part thereof.

"Essentially identical" means that the dsRNA sequence may also have insertions, deletions and single point mutations, in comparison with the ε-cyclase target sequence, and nevertheless causes an efficient reduction in expression. The homology between the sense strand of an inhibitory dsRNA and at least part of the sense-RNA transcript of an ε-cyclase gene or between the antisense strand the complementary strand of an ε-cyclase gene is preferably at least 75%, particularly preferably 80%, very particularly preferably at least 90%, most preferably 100%.

A sequence identity of 100% between dsRNA and an ε-cyclase gene transcript is not absolutely necessary in order to cause an efficient reduction in ε-cyclase expression. As a consequence, it is advantageous that the process is tolerant to sequence deviations as may be present due to genetic mutations, polymorphisms or evolutionary divergences. Thus it is possible, for example, by using the dsRNA generated starting from the ε-cyclase sequence of the first organism, to suppress ε-cyclase expression in a second organism. For this purpose, the dsRNA preferably comprises sequence regions of ε-cyclase gene transcripts corresponding to conserved regions. Said conserved regions may be readily derived from sequence comparisons.

Alternatively, an "essential identical" dsRNA may also be defined as a nucleic acid sequence which is capable of hybridizing with part of an ε-cyclase gene transcript, for example in 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA at 50°C or 70°C for 12 to 16 h).

"Essentially complementary" means that the antisense-RNA strand may also have insertions, deletions and single point mutations, in comparison with the complement of the sense-RNA strand. The

homology between the antisense-RNA strand and the complement of the sense-RNA strand is preferably at least 80%, particularly preferably at least 90%, very particularly preferably at least 95%, most preferably 100%.

Preference is given to transforming the plant with an ε-cyclase dsRNA by using a nucleic acid construct which is introduced into said plant and which is transcribed in said plant into the ε-cyclase dsRNA.

The present invention therefore also relates to a nucleic acid construct transcribable into

a) a sense-RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least part of the sense-RNA ε-cyclase transcript, and

b) an antisense-RNA strand which is essentially, preferably fully, complementary to the RNA sense strand under a).

These nucleic acid constructs are also referred to as expression cassettes or expression vectors hereinbelow.

In a further embodiment, the ε-cyclase dsRNA comprises

a) a sense-RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least part of the sense-RNA transcript of the promoter region of an ε-cyclase gene, and

b) an antisense-RNA strand which is essentially, preferably fully, complementary to the RNA sense strand under a).

Preferably, the promoter region of an ε-cyclase means a sequence according to SEQ ID No. 13 or a part thereof.

The corresponding nucleic acid construct to be used preferably for transformation of the plants comprises

a) a sense-DNA strand which is essentially identical to at least part of the promoter region of an ε-cyclase gene, and

b) an antisense-DNA strand which is essentially, preferably fully, complementary to the DNA sense strand under a).

The ε-cyclase dsRNA sequences and in particular expression cassettes thereof for reducing the ε-cyclase activity, in particular for *Tagetes erecta*, are prepared by using particularly preferably the following partial sequences:

SEQ ID No. 6: sense fragment of the 5'-terminal region of ε-cyclase

SEQ ID No. 7: antisense fragment of the 5'-terminal region of ε-cyclase

SEQ ID No. 8: sense fragment of the 3'-terminal region of ε-cyclase

SEQ ID No. 9: antisense fragment of the 3'-terminal region of ε-cyclase

SEQ ID No. 13: sense fragment of the ε-cyclase promoter

SEQ ID No. 14: antisense fragment of the ε-cyclase promoter

The dsRNA may consist of one or more strands of polyribonucleotides. In order to achieve the same purpose, it is of course also possible to introduce a plurality of individual dsRNA molecules which in each case comprise one of the ribonucleotide sequence sections defined above into the cell or the organism.

The double-stranded dsRNA structure may be formed starting from two complementary, separate RNA strands or, preferably, starting from a single, self-complementary RNA strand. In the latter case, the sense-RNA and antisense-RNA strands are preferably covalently connected to one another in the form of an inverted repeat.

As described, for example, in WO 99/53050, the dsRNA may also comprise a hairpin structure through connection of sense and antisense strands by a connecting sequence ("linker"; for example an intron). The self-complementary dsRNA structures are preferred, because they require merely expression of one RNA sequence and comprise the complementary RNA strands always in an equimolar ratio. The connecting sequence is preferably an intron

(e.g. an intron of the potato ST-LS1 gene; Vancanneyt GF et al. (1990) Mol Gen Genet 220(2):245-250).

The nucleic acid sequence coding for a dsRNA may include further elements such as, for example, transcription termination signals or polyadenylation signals.

However, if the dsRNA is directed against the promoter sequence of an ε-cyclase, it preferably does not comprise any transcription termination signals or polyadenylation signals. This enables the dsRNA to be retained in the nucleus of the cell and prevents the dsRNA from spreading throughout the plant.

If the two strands of the dsRNA are to be assembled in a cell or plant, this may take place in the following way, for example:

a) transformation of the cell or plant with a vector which comprises both expression cassettes,

b) cotransformation of the cell or plant with two vectors, one including the expression cassettes with the sense strand, the other one including the expression cassettes with the antisense strand.

c) crossing of two individual plant lines, one comprising the expression cassettes with the sense strand, the other one comprising the expression cassettes with the antisense strand.

Formation of the RNA duplex may be initiated either outside or inside the cell.

The dsRNA may be synthesized either in vivo or in vitro. For this purpose, it is possible to put a DNA sequence coding for a dsRNA into an expression cassette under the control of at least one genetic control element (such as, for example, a promoter). Polyadenylation is unnecessary, nor need any elements be present to initiate translation. The expression cassette for the MP dsRNA is preferably present on the transformation construct or the transformation vector.

In a preferred embodiment, genetically modified plants are used whose flowers have the lowest rate of expression of an ε-cyclase.

This is preferably achieved by reducing the ε-cyclase activity in a flower-specific, particularly preferably petal-specific, manner.

In the particularly preferred embodiment described above, this is achieved by the ε-cyclase dsRNA sequences being transcribed under the control of a flower-specific promoter or, even more preferably, under the control of a petal-specific promoter.

Therefore, in a particularly preferred embodiment, expression of the dsRNA is carried out starting from an expression construct under the functional control of a flower-specific promoter, particularly preferably under the control of the promoter described by SEQ ID No. 10 or a functionally equivalent part thereof.

The expression cassettes coding for the antisense strand and/or the sense strand of an ε-cyclase dsRNA or for the self-complementary strand of said dsRNA are for this purpose preferably inserted into a transformation vector and introduced into the plant cell using the processes described below. Stable insertion into the genome is advantageous for the process of the invention.

The dsRNA may be introduced in an amount which makes at least one copy possible per cell. Larger amounts (e.g. at least 5, 10, 100, 500 or 1000 copies per cell) may, if appropriate, cause a more efficient reduction.

The methods of dsRNA, cosuppression by means of sense RNA and VIGS (virus-induced gene silencing) are also referred to as post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS).

In a preferred embodiment of the process of the invention, the plant used is a plant selected from the families Ranunculaceae, Berberidaceae, Papaveraceae, Cannabaceae, Rosaceae, Fabaceae, Linaceae, Vitaceae, Brassicaceae, Cucurbitaceae, Primulaceae, Caryophyllaceae, Amaranthaceae, Gentianaceae, Geraniaceae, Caprifoliaceae, Oleaceae, Tropaeolaceae, Solanaceae, Scrophulariaceae, Asteraceae, Liliaceae, Amaryllidaceae, Poaceae, Orchidaceae, Malvaceae, Illiaceae or Lamiaceae.

Particular preference is given to using as plant a plant selected from the plant genera Marigold, Tagetes, Acacia, Aconitum, Adonis, Arnica, Aquilegia, Aster, Astragalus, Bignonia, Calendula, Caltha, Campanula, Canna, Centaurea, Cheiranthus, Chrysanthemum, Citrus, Crepis, Crocus, Curcurbita, Cytisus, Delonia, Delphinium, Dianthus, Dimorphotheca, Doronicum, Eschscholtzia, Forsythia, Fremontia, Gazania, Gelsemium, Genista, Gentiana, Geranium, Gerbera, Geum, Grevilla, Helenium, Helianthus, Hepatica, Heracleum, Hibiscus, Heliopsis, Hypericum, Hypochoeris, Impatiens, Iris, Jacaranda, Kerria, Laburnum, Lathyrus, Leontodon, Lilium, Linum, Lotus, Lycopersicon, Lysimachia, Maratia, Medicago, Mimulus, Narcissus, Oenothera, Osmanthus, Petunia, Photinia, Physalis, Phyteuma, Potentilla, Pyracantha, Ranunculus, Rhododendron, Rosa, Rudbeckia, Senecio, Silene, Silphium, Sinapsis, Sorbus, Spartium, Tecoma, Torenia, Tragopogon, Trollius, Tropaeolum, Tulipa, Tussilago, Ulex, Viola or Zinnia.

Very particular preference is given to using as plant a plant selected from the plant species Marigold, Tagetes erecta or Tagetes patula.

In the process of the invention for preparing zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof, the step of culturing the genetically modified plants, also referred to as transgenic plants hereinbelow, is preferably followed by harvesting the plants and isolating zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof from the plant, particularly preferably from the petals of the plants.

The transgenic plants are grown on nutrient media in a manner known per se and harvested accordingly.

Zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof are isolated from the harvested petals in a manner known per se, for example by drying and subsequent extraction and, if appropriate, further chemical or physical purification processes such as, for example, precipitation methods, crystallography, thermal separation processes such as rectification processes or physical separation processes such as chromatography, for example. For example, zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof are preferably isolated from the petals by organic solvents such as acetone, hexane, ether or methyl tert-butyl ether.

Further processes for isolating ketocarotenoids, in particular from petals, are described, for example, in Egger and Kleinig (Phytochemistry (1967) 6, 437-440) and Egger (Phytochemistry (1965) 4, 609-618).

The biosynthetic intermediates and/or secondary products of zeaxanthin are preferably selected from the group consisting of lycopene, β-carotene, astaxanthin, canthaxanthin, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, adonirubin and adonixanthin, violaxanthin, antheraxanthin, neoxanthin, capsorubin, capsanthin.

Biosynthetic intermediates of zeaxanthin mean carotenoids which, in the biosynthesis diagram, are on the biochemical pathway to zeaxanthin. Said intermediates are preferably lycopene and/or β-carotene.

Biosynthetic secondary products of zeaxanthin mean carotenoids which, in the biosynthesis diagram, derive from zeaxanthin, such as, for example, antheraxanthin, violaxanthin and neoxanthin. However, biosynthetic secondary products of zeaxanthin also mean, in particular, those carotenoids which can be derived from zeaxanthin and its intermediates biosynthetically by introducing further enzymic activities into the plant, for example.

For example, by bringing about a ketolase activity in genetically modified plants, for example by introducing nucleic acids encoding a ketolase into a parent plant, it is possible for the genetically modified plant to be enabled to produce, starting from carotenoids of the β-carotenoid pathway, such as, for example, β-carotene or zeaxanthin, ketocarotenoids such as, for example, astaxanthin, canthaxanthin, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, adonirubin or adonixanthin.

Therefore, biosynthetic secondary products of zeaxanthin also mean in particular astaxanthin, canthaxanthin, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, adonirubin or adonixanthin.

A particularly preferred zeaxanthin secondary product is astaxanthin.

The present invention also refers to a process for preparing genetically modified plants, wherein expression cassettes comprising a nucleic acid construct described above are introduced into a parent plant.

The expression cassettes comprise regulatory signals, i.e. regulating nucleic acid sequences which control the expression of the coding sequence in the host cell. In a preferred embodiment, an expression cassette comprises a promoter upstream, i.e. at the 5' end of the coding sequence, and a polyadenylation signal downstream, i.e. at the 3' end, and, where appropriate, further regulatory elements which are operatively linked to the coding sequence, located in between, for at least one of the genes described above. Operative linkage means the sequential arrangement of promoter, coding sequence, terminator and, where appropriate, further regulatory elements in such a way that each of the regulatory elements is able to carry out its function as intended in the expression of the coding sequence.

The preferred nucleic acid constructs, expression cassettes and vectors for plants and processes for producing transgenic plants, and the transgenic plants themselves, are described by way of example below.

The sequences which are preferred for the operative linkage, but are not restricted thereto, are targeting sequences to ensure the subcellular localization in the apoplast, in the vacuole, in plastids, in the mitochondrion, in the endoplasmic reticulum (ER), in the cell nucleus, in elaioplasts or other compartments and translation enhancers such as the 5' leader sequence from tobacco mosaic virus (Gallie et al., Nucl. Acids Res. 15 (1987), 8693 -8711).

A suitable promoter for the expression cassette is in principle any promoter able to control the expression of foreign genes in plants.

"Constitutive" promoter means promoters which ensure expression in numerous, preferably all, tissues over a relatively wide period during development of the plant, preferably at all times during development of the plant.

Preferably used is, in particular, a plant promoter or a promoter derived from a plant virus. Particular preference is given to the CaMV promoter of the 35S transcript of cauliflower mosaic virus (Franck et al. (1980) Cell 21:285-294; Odell et al. (1985) Nature 313:810-812; Shewmaker et al. (1985) Virology 140:281-288;

Gardner et al. (1986) Plant Mol Biol 6:221-228), or the 19S CaMV promoter (US 5,352,605; WO 84/02913; Benfey et al. (1989) EMBO J 8:2195-2202).

A further suitable constitutive promoter is the pds promoter (Pecker et al. (1992) Proc. Natl. Acad. Sci USA 89: 4962-4966) or the rubisco small subunit (SSU) promoter (US 4,962,028), the legumin B promoter (GenBank Acc. No. X03677), the agrobacterium nopaline synthase promoter, the TR dual promoter, the agrobacterium OCS (octopine synthase) promoter, the ubiquitin promoter (Holtorf S et al. (1995) Plant Mol Biol 29:637-649), the ubiquitin 1 promoter (Christensen et al. (1992) Plant Mol Biol 18:675-689; Bruce et al. (1989) Proc Natl Acad Sci USA 86:9692-9696), the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (US 5,683,439), the promoters of the vacuolar ATPase subunits or the promoter of a proline-rich protein from wheat (WO 91/13991), the Pnit promoter (Y07648.L, Hillebrand et al. (1998), Plant. Mol. Biol. 36, 89-99, Hillebrand et al. (1996), Gene, 170, 197-200) and further promoters of genes whose constitutive expression in plants is known to the skilled worker.

The expression cassettes may also comprise a chemically inducible promoter (review article: Gatz et al. (1997) Annu Rev Plant Physiol Plant Mol Biol 48:89-108) by which expression of the ketolase gene in the plant can be controlled at a particular time. Promoters of this type, such as, for example, the PRP1 promoter (Ward et al. (1993) Plant Mol Biol 22:361-366), a salicylic acid-inducible promoter (WO 95/19443), a benzenesulfonamide-inducible promoter (EP 0 388 186), a tetracycline-inducible promoter (Gatz et al. (1992) Plant J 2:397-404), an abscisic acid-inducible promoter (EP 0 335 528) or an ethanol- or cyclohexanone-inducible promoter (WO 93/21334), can likewise be used.

Promoters which are further preferred are those induced by biotic or abiotic stress, such as, for example, the pathogen-inducible promoter of the PRP1 gene (Ward et al. (1993) Plant Mol Biol 22:361-366), the heat-inducible tomato hsp70 or hsp80 promoter (US 5,187,267), the cold-inducible potato alpha-amylase promoter (WO 96/12814), the light-inducible PPDK promoter or the wound-induced pinII promoter (EP375091).

Pathogen-inducible promoters include those of genes which are induced as a result of pathogen attack, such as, for example, genes of PR proteins, SAR proteins, β-1,3-glucanase, chitinase etc. (for example Redolfi et al. (1983) Neth J Plant Pathol

89:245-254; Uknes, et al. (1992) The Plant Cell 4:645-656; Van Loon (1985) Plant Mol Viral 4:111-116; Marineau et al. (1987) Plant Mol Biol 9:335-342; Matton et al. (1987) Molecular Plant-Microbe Interactions 2:325-342; Somssich et al. (1986) Proc Natl Acad Sci USA 83:2427-2430; Somssich et al. (1988) Mol Gen Genetics 2:93-98; Chen et al. (1996) Plant J 10:955-966; Zhang and Sing (1994) Proc Natl Acad Sci USA 91:2507-2511; Warner, et al. (1993) Plant J 3:191-201; Siebertz et al. (1989) Plant Cell 1:961-968 (1989).

Also included as wound-inducible promoters such as that of the pinII gene (Ryan (1990) Ann Rev Phytopath 28:425-449; Duan et al. (1996) Nat Biotech 14:494-498), of the wun1 and wun2 genes (US 5,428,148), of the win1 and win2 genes (Stanford et al. (1989) Mol Gen Genet 215:200-208), of systemin (McGurl et al. (1992) Science 255:1570-1573), of the WIP1 gene (Rohmeier et al. (1993) Plant Mol Biol 22:783-792; Ekelkamp et al. (1993) FEBS Letters 323:73-76), of the MPI gene (Corderok et al. (1994) The Plant J 6(2):141-150) and the like.

Examples of further suitable promoters are fruit ripening-specific promoters such as, for example, the tomato fruit ripening-specific promoter (WO 94/21794, EP 409 625). Development-dependent promoters include some of the tissue-specific promoters because the formation of some tissues naturally depends on development.

Further particularly preferred promoters are those which ensure expression in tissues or parts of plants in which, for example, the biosynthesis of ketocarotenoids or precursors thereof takes place. Preferred examples are promoters having specificities for anthers, ovaries, petals, sepals, flowers, leaves, stalks and roots and combinations thereof.

Examples of promoters specific for tubers, storage roots or roots are the patatin promoter class I (B33) or the potato cathepsin D inhibitor promoter.

Examples of leaf-specific promoters are the promoter of the potato cytosolic FBPase (WO 97/05900), the rubisco (ribulose-1,5-bisphosphate carboxylase) SSU promoter (small subunit) or the potato ST-LSI promoter (Stockhaus et al. (1989) EMBO J 8:2445-2451).

Examples of flower-specific promoters are the phytoene synthase promoter (WO 92/16635), the promoter of the P-rr gene (WO 98/22593) or, particularly preferably, the modified version,

AP3P, of the flower-specific *Arabidopsis thaliana* AP3 promoter (AL132971: nucleotide region 9298-10200; Hill et al. (1998) Development 125: 1711-1721).

Examples of anther-specific promoters are the 5126 promoter (US 5,689,049, US 5,689,051), the glob-1 promoter or the g-zein promoter.

Further promoters suitable for expression in plants are described in Rogers et al. (1987) Meth in Enzymol 153:253-277; Schardl et al. (1987) Gene 61:1-11 and Berger et al. (1989) Proc Natl Acad Sci USA 86:8402-8406.

All of the promoters described in the present application usually enable the double-stranded ε-cyclase ribonucleic acid sequences to be expressed in the plants of the invention.

Particularly preferred in the process of the invention and in the genetically modified plants of the invention are flower-specific promoters.

An expression cassette is preferably produced by fusing a suitable promoter to a nucleic acid sequence, described above, transcribing a double-stranded ε-cyclase ribonucleic acid sequence, and preferably to a nucleic acid which is inserted between promoter and nucleic acid sequence and which codes for a plastid-specific transit peptide, and to a polyadenylation signal by conventional recombination and cloning techniques as described, for example in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

The preferably inserted nucleic acids encoding a plastid transit peptide ensure localization in plastids and, in particular, in chromoplasts.

The particularly preferred transit peptide is derived from the *Nicotiana tabacum* plastid transketolase or another transit peptide (e.g. the transit peptide of the small subunit of rubisco (rbcS) or of the ferredoxin NADP oxidoreductase, as well as the

isopentenyl-pyrophosphate isomerase 2) or its functional equivalent.

Particular preference is given to nucleic acid sequences of three cassettes of the plastid transit peptide of the tobacco plastid transketolase in three reading frames as KpnI/BamHI fragments with an ATG codon in the NcoI cleavage site:

pTP09

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG
TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGGGA
TCC_BamHI

pTP10

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG
TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGCTG
GATCC_BamHI

pTP11

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG
TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGGGG
ATCC_BamHI

Further examples of a plastid transit peptide are the transit peptide of the Arabidopsis thaliana plastid isopentenyl-pyrophosphate isomerase 2 (IPP-2) and the transit peptide of the small subunit of ribulose-bisphosphate carboxylase (rbcS) from pea (Guerineau, F, Woolston, S, Brooks, L, Mullineaux, P (1988) An expression cassette for targeting foreign proteins into the chloroplasts. Nucl. Acids Res. 16: 11380).

The nucleic acids of the invention can be prepared synthetically or obtained naturally or comprise a mixture of synthetic and natural nucleic acid constituents, and consist of various heterologous gene sections from different organisms.

Examples of a terminator are the 35S terminator (Guerineau et al. (1988) Nucl Acids Res. 16: 11380), the nos terminator (Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman HM. Nopaline synthase: transcript mapping and DNA sequence. J Mol Appl Genet. 1982;1(6):561-73) or the ocs terminator (Gielen, J, de Beuckeleer, M, Seurinck, J, Debroek, H, de Greve, H, Lemmers, M, van Montagu, M, Schell, J (1984) The complete sequence of the TL-DNA of the Agrobacterium tumefaciens plasmid pTiAch5. EMBO J. 3: 835-846).

It is furthermore possible to employ manipulations which provide appropriate restriction cleavage sites or delete the redundant DNA or restriction cleavage sites. It is possible in relation to insertions, deletions or substitutions, such as, for example, transitions and transversions, to use *in vitro* mutagenesis, primer repair, restriction or ligation.

It is possible with suitable manipulations, such as, for example, restriction, chewing back or filling in of overhangs for blunt ends, to provide complementary ends of the fragments for ligation.

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which essentially correspond to T-DNA polyadenylation signals from Agrobacterium tumefaciens, especially of gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984), 835 ff) or functional equivalents.

The transfer of nucleic acid sequences into the genome of a plant is referred to as transformation.

It is possible to use for this purpose methods known per se for the transformation and regeneration of plants from plant tissues or plant cells for transient or stable transformation.

Suitable methods for transforming plants are protoplast transformation by polyethylene glycol-induced DNA uptake, the biolistic method using the gene gun - called the particle bombardment method, electroporation, incubation of dry embryos in DNA-containing solution, microinjection and gene transfer mediated by Agrobacterium described above. Said processes are described, for example, in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press

(1993), 128-143 and in Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225.

The construct to be expressed is preferably cloned into a vector which is suitable for transforming Agrobacterium tumefaciens, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984), 8711) or particularly preferably, pSUN2, pSUN3, pSUN4 or pSUN5 (WO 02/00900).

Agrobacteria transformed with an expression cassette can be used in a known manner for transforming plants, e.g. bathing wounded leaves or pieces of leaf in a solution of agrobacteria and subsequently cultivating in suitable media.

For the preferred production of genetically modified plants, also referred to as transgenic plants hereinafter, the fused expression cassette is cloned into a vector, for example pBin19 or, in particular, pSUN5, which is suitable for transforming *Agrobacterium tumefaciens*.

Agrobacteria transformed with such a vector can then be used in a known manner for transforming plants, in particular crop plants, for example by bathing wounded leaves or pieces of leaf in a solution of agrobacteria and subsequently cultivating in suitable media.

The transformation of plants by agrobacteria is disclosed inter alia in F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press, 1993, pages 15-38. Transgenic plants which comprise a gene, integrated into the expression cassette for expression of a nucleic acid encoding a ketolase can be regenerated in a known manner from the transformed cells of the wounded leaves or pieces of leaf.

To transform a host cell with a double-stranded ε-cyclase ribonucleic acid sequence, an expression cassette is incorporated and inserted into a recombinant vector whose vector DNA comprises additional functional regulatory signals, for example sequences for replication or integration. Suitable vectors are described inter alia in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), chapters 6/7, pages 71-119 (1993).

Using the recombination and cloning techniques quoted above, the expression cassettes can be cloned into suitable vectors which make replication thereof possible for example in *E. coli*. Suitable cloning vectors are, inter alia, pJIT117 (Guerineau et

al. (1988) Nucl. Acids Res.16 :11380), pBR322, pUC series, M13mp series and pACYC184. Binary vectors which are able to replicate both in *E. coli* and in agrobacteria are particularly suitable.

The invention further relates to the genetically modified plants which, in comparison with the wild type, have a reduced ε-cyclase activity caused by double-stranded ε-cyclase ribonucleic acid sequences.

As mentioned above, the genetically modified plant comprises, in a particular embodiment, an RNA which has a double-stranded structural region and comprises, in said region, a nucleic acid sequence which

a) is identical to at least part of the ε-cyclase transcript intrinsic to said plant and/or

b) is identical to at least part of the ε-cyclase-promoter sequence intrinsic to said plant.

Preference is given to genetically modified plants selected from the families Ranunculaceae, Berberidaceae, Papaveraceae, Cannabaceae, Rosaceae, Fabaceae, Linaceae, Vitaceae, Brassicaeae, Cucurbitaceae, Primulaceae, Caryophyllaceae, Amaranthaceae, Gentianaceae, Geraniaceae, Caprifoliaceae, Oleaceae, Tropaeolaceae, Solanaceae, Scrophulariaceae, Asteraceae, Liliaceae, Amaryllidaceae, Poaceae, Orchidaceae, Malvaceae, Illiaceae or Lamiaceae.

Particular preference is given to genetically modified plants selected from the plant genera Marigold, Tagetes, Acacia, Aconitum, Adonis, Arnica, Aquilegia, Aster, Astragalus, Bignonia, Calendula, Caltha, Campanula, Canna, Centaurea, Cheiranthus, Chrysanthemum, Citrus, Crepis, Crocus, Curcurbita, Cytisus, Delonia, Delphinium, Dianthus, Dimorphoteca, Doronicum, Escholtzia, Forsythia, Fremontia, Gazania, Gelsemium, Genista, Gentiana, Geranium, Gerbera, Geum, Grevilla, Helenium, Helianthus, Hepatica, Heracleum, Hisbiscus, Heliopsis, Hyperricum, Hypochoeris, Impatiens, Iris, Jacaranda, Kerria, Laburnum, Lathyrus, Leontodon, Lilium, Linum, Lotus, Lycopersicon, Lysimachia, Maratia, Medicago, Mimulus, Narcissus, Oenothera, Osmanthus, Petunia, Photinia, Physalis, Phyteuma, Potentilla, Pyracantha, Ranunculus, Rhododendron, Rosa, Rudbeckia, Senecio, Silene, Silphium, Sinapsis, Sorbus, Spartium,

Tecoma, Torenia, Tragopogon, Trollius, Tropaeolum, Tulipa, Tussilago, Ulex, Viola or Zinnia.

Very particular preference is given to genetically modified plants selected from the plant genera Marigold, Tagetes erecta or Tagetes patula.

The present invention furthermore relates to the transgenic plants, to the propagation material thereof and also to the plant cells, tissues or parts thereof, in particular to the petals thereof.

The genetically modified plants may, as described above, be used for preparing zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof, in particular for preparing lycopene, β-carotene, astaxanthin, canthaxanthin, echinenone 3-hydroxyechinenone, 3'-hydroxyechinenone, adonirubin or adonixanthin, and in particular for preparing astaxanthin.

The genetically modified plants of the invention have, in comparison with the wild type, an increased content of at least one carotenoid selected from the group consisting of zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof.

In this case, an increased content also means a ketocarotenoid, or astaxanthin, content which has been brought about.

Genetically modified plants of the invention, which have an increased content of zeaxanthin and/or biosynthetic intermediates and/or secondary products thereof and which are consumable by humans and animals, may also be used, for example, directly or after processing known per se as foodstuffs or feedstuffs or as feed supplements and food supplements. The genetically modified plants may also be used for preparing carotenoid-containing extracts of said plants and/or for preparing feed supplements and food supplements.

The genetically modified plants may also be used as ornamental plants in the field of horticulture.

The invention will now be illustrated by the following examples, without being limited thereto:

General experimental conditions:
Sequence analysis of recombinant DNA

Recombinant DNA molecules were sequenced using a laser fluorescence DNA sequencer from Licor (sold by MWG Biotech, Ebersbach, Germany), according to the method of Sanger (Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977), 5463-5467).

Example 1: Preparation of a cloning vector for preparing double-stranded ε-cyclase ribonucleic acid sequence expression cassettes for flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes erecta*

Inverted repeat transcripts consisting of epsilon-cyclase fragments were expressed in *Tagetes erecta* under the control of a modified version, AP3P, of the flower-specific *Arabidopsis thaliana* promoter AP3 (AL132971: Nucleotide region 9298-10200; Hill et al. (1998) Development 125: 1711-1721).

The inverted repeat transcript includes in each case a fragment in the correct orientation (sense fragment) and a sequence-identical fragment in the opposite orientation (antisense fragment) which are connected to one another by a functional intron, the PIV2 intron of the potato ST-LH1 gene (Vancanneyt G. et al. (1990) Mol Gen Genet 220: 245-50).

The cDNA coding for the *Arabidopsis thaliana* AP3 promoter (-902 to +15) was prepared by means of PCR using genomic DNA (isolated from *Arabidopsis thaliana* by a standard method) and the primers PR7 (SEQ ID No. 15) and PR10 (SEQ ID No. 18).

The PCR conditions were as follows:

The PCR for amplification of the DNA encoding the AP3 promoter fragment (-902 to +15) was carried out in a 50 µl reaction mixture containing:

- 1 µl *A.thaliana* genomic DNA (diluted 1:100, prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID No. 15)
- 0.2 mM PR10 (SEQ ID No. 18)
- 5 µl 10X PCR buffer (Stratagene)
- 0.25 µl Pfu polymerase (Stratagene)

- 28.8 µl distilled water

The PCR was carried out under the following cycle conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The 922 bp amplicon was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) by using standard methods, resulting in the plasmid pTAP3. Sequencing of the pTAP3 clone confirmed a sequence which differs from the published AP3 sequence (AL132971, nucleotide region 9298-10200) merely in an insertion (a G in position 9765 of the AL132971 sequence) and a base substitution (G for A in position 9726 of the AL132971 sequence) (position 33: T for G, position 55: T for G). These nucleotide differences were reproduced in an independent amplification experiment and thus represent the nucleotide sequence in the *Arabidopsis thaliana* plant used.

The modified version, AP3P, was prepared by means of recombinant PCR using the pTAP3 plasmid. The region 10200-9771 was amplified using the primers PR7 (SEQ ID No. 15) and PR9 (SEQ ID No. 17) (amplicon A7/9), the region 9526-9285 was amplified using PR8 (SEQ ID No. 16) and PR10 (SEQ ID No. 18) (amplicon A8/10).

The PCR conditions were as follows:

The PCR reactions for amplification of the DNA fragments coding for the regions 10200-9771 and 9526-9285 of the AP3 promoter were carried out in 50 µl reaction mixtures containing:

- 100 ng AP3 amplicon (described above)
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID No. 15) or PR8 (SEQ ID No. 16)
- 0.2 mM PR9 (SEQ ID No. 17) or PR10 (SEQ ID No. 18)
- 5 µl 10 X PCR buffer (Stratagene)
- 0.25 µl Pfu Taq polymerase (Stratagene)
- 28.8 µl distilled water

The PCR was carried out under the following cycle conditions:

1 X	94°C	2 minutes
35 X	94°C	1 minute
	50°C	2 minutes
	72°C	3 minutes
1 X	72°C	10 minutes

The recombinant PCR includes annealing of the amplicons A7/9 and A8/10 which overlap over a sequence of 25 nucleotides, completion to give a double strand and subsequent amplification. This results in a modified version of the AP3 promoter, AP3P, in which positions 9670-9526 have been deleted. The two amplicons A7/9 and A8/10 were denatured (5 min at 95°C) and annealed (slowly cooling to 40°C at room temperature) in a 17.6 µl reaction mixture containing:

- 0.5 µg A7/9
- 0.25 µg A8/10

The 3' ends were filled in (30 min at 30°C) in a 20 µl reaction mixture containing:

- 17.6 µl A7/9 and A8/10 annealing reactions (prepared as described above)
- 50 µM dNTPs
- 2 µl 1 X Klenow buffer
- 2 U Klenow enzyme

The nucleic acid coding for the modified promoter version, AP3P, was amplified by means of PCR using a sense-specific primer (PR7 SEQ ID No. 15) and an antisense-specific primer (PR10 SEQ ID No. 18).

The PCR conditions were as follows:

The PCR for amplification of the AP3P fragment was carried out in a 50 µl reaction mixture containing:

- 1 µl annealing reaction (prepared as described above)
- 0.25 mM dNTPs

- 0.2 mM PR7 (SEQ ID No. 15)
- 0.2 mM PR10 (SEQ ID No. 18)
- 5 µl 10 X PCR buffer (Stratagene)
- 0.25 µl Pfu Taq polymerase (Stratagene)
- 28.8 µl distilled water

The PCR was carried out under the following cycle conditions:

1 X	94°C	2 minutes
35 X	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1 X	72°C	10 minutes

The PCR amplification with PR7, SEQ ID No. 15 and PR10 SEQ ID No. 18 resulted in a 778 bp fragment coding for the modified promoter version, AP3P. The amplicon was cloned into the cloning vector pCR2.1 (Invitrogen). Sequencing reactions using the primers T7 and M13 confirmed a sequence identical to the sequence AL132971, region 10200-9298, with the internal region 9285-9526 having been deleted. This clone was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

The cloning was carried out by isolating the 771 bp SacI-HindIII fragment from pTAP3P and ligation into the SacI-HindIII-cut pJIT117 vector. The clone which contains the promoter AP3P instead of the original promoter d35S is denoted pJAP3P.

A DNA fragment containing the PIV2 intron of the ST-LS1 gene was prepared by means of PCR using p35SGUS INT plasmid DNA (Vancanneyt G. et al.(1990) Mol Gen Genet 220: 245-50) and the primers PR40 (Seq ID No. 20) and PR41 (Seq ID No. 21).

The PCR conditions were as follows:

The PCR for amplification of the PIV2 intron sequence of the ST-LS1 gene was carried out in a 50 µl reaction mixture containing:

- 1 µl p35SGUS INT
- 0.25 mM dNTPs

- 0.2 µM PR40 (SEQ ID No. 20)
- 0.2 µM PR41 (SEQ ID No. 21)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR was carried out under the following cycle conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	53°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

PCR amplification using PR40 and PR41 resulted in a 206 bp fragment. Using standard methods, the amplicon was cloned into the PCR cloning vector pBluntII (Invitrogen), resulting in the clone pBluntII-40-41. Sequencing reactions of this clone, using the primer SP6, confirmed a sequence which is identical to the corresponding sequence of the p35SGUS INT vector.

This clone was therefore for cloning into the pJAP3P vector (described above).

The cloning was carried out by isolating the 206 bp SalI-BamHI fragment from pBluntII-40-41 and ligation with the SalI-BamHI-cut pJAP3P vector. The clone which contains the PIV2 intron of the ST-LS1 gene in the correct orientation, downstream of the 3' end of the rbcs transit peptide, is denoted pJAI1 and is suitable for preparation of expression cassettes for flower-specific expression of inverted repeat transcripts.

In figure 2, the *AP3P* fragment includes the modified AP3P promoter (771 bp), the *rbcs* fragment includes the pea rbcS transit peptide (204 bp), the *intron* fragment includes the PIV2 intron of the potato ST-LS1 gene, and the *term* fragment (761 bp) includes the CaMV polyadenylation signal.

Example 2: Preparation of inverted repeat expression cassettes for flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes erecta* (directed against the 5' region of epsilon-cyclase cDNA)

The nucleic acid containing the 5'-terminal 435 bp region of epsilon-cyclase cDNA (GenBank accession no. AF251016) was amplified by means of polymerase chain reaction (PCR) from *Tagetes erecta* cDNA by using a sense-specific primer (PR42 SEQ ID No. 22) and an antisense-specific primer (PR43 SEQ ID No. 23). The 5'-terminal 435 bp region of the *Tagetes erecta* epsilon-cyclase cDNA is composed of 138 bp of 5'-untranslated sequence (5'UTR) and 297 bp of the coding region corresponding to the N terminus.

To prepare total RNA from Tagetes flowers, 100 mg of the frozen, pulverized flowers were transferred to a reaction vessel and taken up in 0.8 ml of Trizol buffer (LifeTechnologies). The suspension was extracted with 0.2 ml of chloroform. After centrifugation at 12 000 g for 15 minutes, the aqueous supernatant was removed and transferred to a new reaction vessel and extracted with one volume of ethanol. The RNA was precipitated with one volume of isopropanol, washed with 75% ethanol and the pellet was dissolved in DEPC water (overnight incubation of water with 1/1000 volume of diethyl pyrocarbonate at room temperature, with subsequent autoclaving). The RNA concentration was determined photometrically. For cDNA synthesis, 2.5 µg of total RNA were denatured at 60°C for 10 min, cooled on ice for 2 min and transcribed into cDNA by means of a cDNA kit (Ready-to-go-you-prime-beads, Pharmacia Biotech) according to the manufacturer's information, using an antisense-specific primer (PR17 SEQ ID No. 19).

The conditions of the subsequent PCR reactions were as follows:

The PCR for amplification of the PR42-PR43 DNA fragment containing the 5'-terminal 435 bp region of epsilon-cyclase was carried out in a 50 µl reaction mixture containing:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR42 (SEQ ID No. 22)
- 0.2 µM PR43 (SEQ ID No. 23)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)

- 28.8 µl distilled water

The PCR for amplification of the PR44-PR45 DNA fragment containing the 5'-terminal 435 bp region of epsilon-cyclase was carried out in a 50 µl reaction mixture containing:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR44 (SEQ ID No. 24)
- 0.2 µM PR45 (SEQ ID No. 25)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR reactions were carried out under the following cycle conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	58°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

PCR amplification using primers PR42 and PR43 resulted in a 443 bp fragment, and PCR amplification using primers PR44 and PR45 resulted in a 444 bp fragment.

The two amplicons, the PR42-PR43 (HindIII-SalI sense) fragment and the PR44-PR45 (EcoRI-BamHI antisense) fragment, were cloned into the PCR-cloning vector pCR-BluntII (Invitrogen), using standard methods. Sequence reactions using the SP6 primer confirmed in each case a sequence identical to the published AF251016 sequence (SEQ ID No. 4), apart from the introduced restriction sites. These clones were therefore used for preparing an inverted repeat construct in the pJAI1 cloning vector (see Example 1).

The first cloning step was carried out by isolating the 444 bp PR44-PR45 BamHI-EcoRI fragment from the pCR-BluntII cloning vector (Invitrogen) and ligation with the BamHI-EcoRI-cut pJAI1 vector. The clone which contains the 5'-terminal epsilon-cyclase region in the antisense orientation is denoted pJAI2. The ligation results in a transcriptional fusion between the

antisense fragment of the 5'-terminal epsilon-cyclase region and the CaMV polyadenylation signal.

The second cloning step is carried out by isolating the 443 bp PR42-PR43 HindIII-SalI fragment from the pCR-BluntII cloning vector (Invitrogen) and ligation with the HindIII-SalI-cut pJAI2 vector. The clone which contains the 435 bp 5'-terminal region of epsilon-cyclase cDNA in the sense orientation is denoted pJAI3. The ligation results in a transcriptional fusion between the AP3P and the sense fragment of the 5'-terminal epsilon-cyclase region.

An inverted repeat expression cassette under the control of the CHRC promoter was prepared by amplifying an CHRC promoter fragment, using petunia genomic DNA (prepared according to standard methods) and the primers PRCHRC5 (SEQ ID No. 42) and PRCHRC3 (SEQ ID No. 43). The amplicon was cloned into the pCR2.1 cloning vector (Invitrogen). Sequencing reactions of the resulting clone pCR2.1-CHRC, using the primers M13 and T7, confirmed a sequence identical to the AF099501 sequence. This clone was therefore used for cloning into the pJAI3 expression vector.

The cloning was carried out by isolating the 1537 bp SacI-HindIII fragment from pCR2.1-CHRC and ligation into the SacI-HindIII-cut pJAI3 vector. The clone which contains the CHRC promoter instead of the original AP3P promoter is denoted pJCI3.

The expression vectors for Agrobacterium-mediated transformation of the AP3P- or CHRC-controlled inverted repeat transcript in *Tagetes erecta* were prepared using the binary vector pSUN5 (WO02/00900).

The expression vector pS5AI3 was prepared by ligating the 2622 bp SacI-XhoI fragment of pJAI3 with the SacI-XhoI-cut pSUN5 vector (Figure 3, construct map).

In Figure 3, the *AP3P* fragment includes the modified AP3P promoter (771 bp), the *5sense* fragment includes the 5' region of *Tagetes erecta* epsilon-cyclase (435 bp) in the sense orientation, the *intron* fragment includes the PIV2 intron of the potato ST-LS1 gene, the *5anti* fragment includes the 5' region of *Tagetes erecta* epsilon-cyclase (435 bp) in the antisense orientation, and the *term* fragment (761 bp) includes the CaMV polyadenylation signal.

The expression vector pS5CI3 was prepared by ligating the 3394 bp SacI-XhoI fragment of pJCI3 with the SacI-XhoI-cut pSUN5 vector (Figure 4, construct map).

In Figure 4, the *CHRC* fragment includes the promoter (1537 bp), the *5sense* fragment includes the 5' region of *Tagetes erecta* epsilon-cyclase (435 bp) in the sense orientation, the *intron* fragment includes the PIV2 intron of the potato ST-LS1 gene, the *5anti* fragment includes the 5' region of *Tagetes erecta* epsilon-cyclase (435 bp) in the antisense orientation, and the *term* fragment (761 bp) includes the CaMV polyadenylation signal.

Example 3: Preparation of an inverted repeat expression cassette for flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes erecta* (directed against the 3' region of epsilon-cyclase cDNA)

The nucleic acid containing the 3'-terminal region (384 bp) of epsilon-cyclase cDNA (GenBank accession no. AF251016) was amplified by means of polymerase chain reaction (PCR) from *Tagetes erecta* cDNA, using a sense-specific primer (PR46 SEQ ID No. 26) and an antisense-specific primer (PR47 SEQ ID No. *27)*. The 3'-terminal region (384 bp) of *Tagetes erecta* epsilon-cyclase cDNA is composed of 140 bp of 3'-untranslated sequence (3'UTR) and 244 bp of the coding region corresponding to the C terminus.

Total RNA was prepared from Tagetes flowers as described in Example 2.

The cDNA synthesis was carried out as described in Example 1, using the antisense-specific primer PR17 (SEQ ID No. 19).

The conditions of the subsequent PCR reactions were as follows:

The PCR for amplification of the PR46-PR457 DNA fragment containing the 3'-terminal 384 bp region of epsilon-cyclase was carried out in a 50 µl reaction mixture containing:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR46 (SEQ ID No. 26)
- 0.2 µM PR47 (SEQ ID No. 27)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)

- 28.8 µl distilled water

The PCR for amplification of the PR48-PR49 DNA fragment containing the 3'-terminal 384 bp region of epsilon-cyclase was carried out in a 50 µl reaction mixture containing:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR48 (SEQ ID No. 28)
- 0.2 µM PR49 (SEQ ID No. 29)
- 5 µl 10 X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR reactions were carried out under the following cycle conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	58°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

PCR amplification using SEQ ID No. 26 and SEQ ID No. 27 resulted in a 392 bp fragment, and PCR amplification using SEQ ID No. 28 and SEQ ID No. 29 resulted in a 396 bp fragment.

The two amplicons, the PR46-PR47 fragment and the PR48-PR49 fragment, were cloned into the PCR-cloning vector pCR-BluntII (Invitrogen), using standard methods. Sequence reactions using the SP6 primer confirmed in each case a sequence identical to the published AF251016 sequence (SEQ ID No. 4), apart from the introduced restriction sites. These clones were therefore used for preparing an inverted repeat construct in the pJAI1 cloning vector (see Example 1).

The first cloning step was carried out by isolating the 396 bp PR48-PR49 BamHI-EcoRI fragment from the pCR-BluntII cloning vector (Invitrogen) and ligation with the BamHI-EcoRI-cut pJAI1 vector. The clone which contains the 3'-terminal epsilon-cyclase region in the antisense orientation is denoted pJAI4. The ligation results in a transcriptional fusion between the

antisense fragment of the 3'-terminal epsilon-cyclase region and the CaMV polyadenylation signal.

The second cloning step is carried out by isolating the 392 bp PR46-PR47 HindIII-SalI fragment from the pCR-BluntII cloning vector (Invitrogen) and ligation with the HindIII-SalI-cut pJAI4 vector. The clone which contains the 392 bp 3'-terminal region of epsilon-cyclase cDNA in the sense orientation is denoted pJAI5. The ligation results in a transcriptional fusion between the AP3P and the sense fragment of the 3'-terminal epsilon-cyclase region.

An expression vector for Agrobacterium-mediated transformation of the AP3P-controlled inverted repeat transcript in Tagetes erecta was prepared using the binary pSUN5 vector (WO02/00900). The expression vector pS5AI5 was prepared by ligating the 2523 bp SacI-XhoI fragment of pJAI5 with the SacI-XhoI-cut pSUN5 vector (Figure 5, construct map).

In Figure 5, the *AP3P* fragment includes the modified AP3P promoter (771 bp), the *sense* fragment includes the 3' region of Tagetes erecta epsilon-cyclase (435 bp) in the sense orientation, the *intron* fragment includes the IV2 intron of the potato ST-LS1 gene, the *anti* fragment includes the 3' region of Tagetes erecta epsilon-cyclase (435 bp) in the antisense orientation, and the *term* fragment (761 bp) includes the CaMV polyadenylation signal.

Example 4: Cloning of the epsilon-cyclase promoter

A 199 bp fragment and, respectively, the 312 bp fragment of the epsilon-cyclase promoter were isolated by two independent cloning strategies, inverse PCR (adapted from Long et al. Proc. Natl. Acad. Sci USA 90: 10370) and TAIL-PCR (Liu Y-G. et al. (1995) Plant J. 8: 457-463), using genomic DNA (isolated by a standard method from *Tagetes erecta*, line "Orangenprinz").

For the inverse PCR approach, 2 µg of genomic DNA were digested with EcoRV and RsaI in a 25 µl reaction mixture, then diluted to 300 µl and religated at 16°C overnight, using 3U of ligase. PCR amplification using the primers PR50 (SEQ ID No. 30) and PR51 (SEQ ID No. 31) produced a fragment which contains, in each case in the sense orientation, 354 bp of epsilon-cyclase cDNA (GenBank Accession AF251016), ligated to 300 bp of the epsilon-cyclase promoter and 70 bp of the 5'-terminal region of epsilon-cyclase cDNA (see Figure 6).

The conditions of the PCR reactions were as follows:

The PCR for amplification of the PR50-PR51 DNA fragment which contains, inter alia, the 312 bp promoter fragment of epsilon-cyclase was carried out in a 50 µl reaction mixture containing:

-	1	µl	ligation mixture (prepared as described above)
-	0.25	mM	dNTPs
-	0.2	µM	PR50 (SEQ ID No. 30)
-	0.2	µM	PR51 (SEQ ID No. 31)
-	5	µl	10X PCR buffer (TAKARA)
-	0.25	µl	R Taq polymerase (TAKARA)
-	28.8	µl	distilled water

The PCR reactions were carried out under the following cycle conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	53°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

PCR amplification using primers PR50 and PR51 resulted in a 734 bp fragment containing, inter alia, the 312 bp promoter fragment of epsilon-cyclase (Figure 6).

The amplicon was cloned into the PCR-cloning vector pCR2.1 (Invitrogen), using standard methods. Sequencing reactions using the primers M13 and T7 produced the sequence SEQ ID No. 11. This sequence was reproduced in an independent amplification experiment and thus represents the nucleotide sequence in the *Tagetes erecta* line "Orangenprinz" used.

For the TAIL-PCR approach, three successive PCR reactions were carried out, using in each case different gene-specific primers (nested primers).

The TAIL1-PCR was carried out in a 20 µl reaction mixture containing:

- 1 ng genomic DNA (prepared as described above)
- 0.2 mM each of dNTPs
- 0.2 µM PR60 (SEQ ID No. 32)
- 0.2 µM AD1 (SEQ ID No. 35)
- 2 µl 10X PCR buffer (TAKARA)
- 0.5 µl R Taq polymerase (TAKARA)
- ad 20 µl distilled water

In this context, AD1 was initially a mixture of primers of the sequences (a/c/g/t)tcga(g/c)t(a/t)t(g/c)g(a/t)gtt.

The PCR reaction TAIL1 was carried out under the following cycle conditions:

1X 93°C: 1 min., 95°C: 1 min.
5X 94°C: 30 sec., 62°C: 1 min., 72°C: 2.5 min.
1X 94°C: 30 sec., 25°C: 3 min., ramp to 72°C in 3 min.
72°C: 2.5 min
15X 94°C: 10 sec., 68°C: 1 min., 72°C: 2.5 min.;
94°C: 10 sec., 68°C: 1 min., 72°C: 2.5 min.;
94°C: 10 sec., 29°C: 1 min., 72°C: 2.5 min.
1X 72°C: 5 min.

The TAIL2-PCR was carried out in a 21 µl reaction mixture containing:

- 1 µl 1:50 dilution of the TAIL1 reaction mixture (prepared as described above)
- 0.8 mM dNTP
- 0.2 µM PR61 (SEQ ID No. 33)
- 0.2 µM AD1 (SEQ ID No. 35)
- 2 µl 10X PCR buffer (TAKARA)
- 0.5 µl R Taq polymerase (TAKARA)
- ad 21 µl distilled water

The PCR reaction TAIL2 was carried out under the following cycle conditions:

12X 94°C: 10 seconds, 64°C: 1 minute, 72°C: 2.5 minutes;
94°C: 10 seconds, 64°C: 1 minute, 72°C: 2.5 minutes;

94°C: 10 seconds, 29°C: 1 minute, 72°C: 2.5 minutes;
1X 72°C: 5 minutes

The TAIL3 PCR was carried out in a 100 µl reaction mixture containing:

- 1 µl 1:10 dilution of the TAIL2 reaction mixture (prepared as described above)
- 0.8 mM dNTP
- 0.2 µM PR63 (SEQ ID No. 34)
- 0.2 µM AD1 (SEQ ID No. 35)
- 10 µl 10X PCR buffer (TAKARA)
- 0.5 µl R Taq polymerase (TAKARA)
- ad 100 µl distilled water

The PCR reaction TAIL3 was carried out under the following cycle conditions:

20X 94°C: 15 seconds, 29°C: 30 seconds, 72°C: 2 minutes
1X 72°C: 5 minutes

PCR amplification using primers PR63 and AD1 resulted in a 280 bp fragment containing, inter alia, the 199 bp promoter fragment of epsilon-cyclase (Figure 7).

The amplicon was cloned into the PCR-cloning vector pCR2.1 (Invitrogen), using standard methods. Sequencing reactions using the primers M13 and T7 produced the sequence SEQ ID No. 12. This sequence is identical to the e-cyclase region within the sequence SEQ ID No. 11, isolated using the IPCR strategy, and thus represents the nucleotide sequence in the *Tagetes erecta* line "Orangenprinz" used.

The pCR2.1 clone which contains the 312 bp fragment (SEQ ID No. 11) of the epsilon-cyclase promoter, isolated by the IPCR strategy, is denoted pTA-ecycP and was used for preparing the IR constructs.

Example 5: Preparation of an inverted repeat expression cassette for flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes erecta* (directed against the promoter region of epsilon-cyclase cDNA).

Inverted repeat transcripts consisting of promoter fragments of epsilon-cyclase were expressed in *Tagetes erecta* under the control of a modified version, AP3P, of the flower-specific *Arabidopsis* promoter AP3 (see Example 1) or of the flower-specific promoter CHRC (GenBank accession no. AF099501). The inverted repeat transcript contains in each case an epsilon-cyclase promoter fragment in the correct orientation (sense fragment) and a sequence-identical epsilon-cyclase promoter fragment in the opposite orientation (antisense fragment) which are connected to one another by a functional intron (see Example 1).

The promoter fragments were prepared by means of PCR using plasmid DNA (pTA-ecycP clone, see Example 4) and the primers PR124 (SEQ ID No. 36) and PR126 (SEQ ID No. 38) and, respectively, the primers PR125 (SEQ ID No. 37) and PR127 (SEQ ID No. 39).

The conditions of the PCR reactions were as follows:

The PCR for amplification of the PR124-PR126 DNA fragment containing the promoter fragment of epsilon-cyclase was carried out in a 50 µl reaction mixture containing:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR124 (SEQ ID No. 36)
- 0.2 µM PR126 (SEQ ID No. 38)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR for amplification of the PR125-PR127 DNA fragment containing the 312 bp promoter fragment of epsilon-cyclase was carried out in a 50 µl reaction mixture containing:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR125 (SEQ ID No. 37)
- 0.2 µM PR127 (SEQ ID No. 39)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)

- 28.8 µl distilled water

The PCR reactions were carried out under the following cycle conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	53°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

PCR amplification using the primers PR124 and PR126 resulted in a 358 bp fragment, and PCR amplification using primers PR125 and PR127 resulted in a 361 bp fragment.

The two amplicons, the PR124-PR126 (HindIII-SalI sense) fragment and the PR125-PR127 (EcoRI-BamHI antisense) fragment, were cloned into the PCR-cloning vector pCR-BluntII (Invitrogen), using standard methods. Sequencing reactions using the SP6 primer confirmed in each case a sequence which is identical to SEQ ID No. 11, apart from the introduced restriction sites. These clones were therefore used for preparing an inverted repeat construct in the pJAI1 cloning vector (see Example 1).

The first cloning step was carried out by isolating the 358 bp PR124-PR126 HindIII-SalI fragment from the pCR-BluntII cloning vector (Invitrogen) and ligation with the BamHI-EcoRI-cut pJAI1 vector. The clone which contains the epsilon-cyclase promoter fragment in the sense orientation is denoted cs43. The ligation causes the sense fragment of the epsilon-cyclase promoter to be inserted between the AP3P promoter and the intron.

The second cloning step was carried out by isolating the 361 bp PR125-PR127 BamHI-EcoRI fragment from the pCR-BluntII cloning vector (Invitrogen) and ligation with the BamHI-EcoRI-cut cs43 vector. The clone which contains the epsilon-cyclase promoter fragment in the antisense orientation is denoted cs44. The ligation produces a transcriptional fusion between the intron and the antisense fragment of the epsilon-cyclase promoter.

An inverted repeat expression cassette under the control of the CHRC promoter was prepared by amplifying a CHRC promoter fragment, using petunia genomic DNA (prepared by standard methods) and the primers PRCHRC3' (SEQ ID No. 43) and PRCHRC5'

(SEQ ID No. 42). The amplicon was cloned into the pCR2.1 cloning vector (Invitrogen). Sequencing reactions of the resulting clone pCR2.1-CHRC, using the M13 and T7 primers, confirmed a sequence identical to the AF099501 sequence. This clone was therefore used for cloning into the expression vector cs44.

The cloning was carried out by isolating the 1537 bp SacI-HindIII fragment of pCR2.1-CHRC and ligation into the SacI-HindIII-cut cs44 vector. The clone which contains the CHRC promoter instead of the original AP3P promoter is denoted cs45.

An inverted repeat expression cassette under the control of two promoters, the CHRC promoter and the AP3P promoter, was prepared by cloning the AP3P promoter in antisense orientation to the 3' terminus of the epsilon-cyclase antisense fragment in cs45. The AP3P promoter fragment of pJAI1 was amplified using the primers PR128 and PR129. The amplicon was cloned into the pCR2.1 cloning vector (Invitrogen). Sequencing using the M13 and T7 primers confirmed a sequence identical to the sequence SEQ ID No. 1. This clone, pCR2.1-AP3PSX, was used for preparing an inverted repeat expression cassette under the control of two promoters.

The cloning was carried out by isolating the 771 bp SalI-XhoI fragment from pCR2.1-AP3PSX and ligation into the XhoI-cut cs45 vector. The clone which contains, 3' of the inverted repeat, the AP3P promoter in the antisense orientation is denoted cs46.

The expression vectors for Agrobacterium-mediated transformation of the AP3P-controlled inverted repeat transcript in Tagetes erecta were prepared using the binary pSUN5 vector (WO02/00900).

The expression vector pS5AI7 was prepared by ligating the 1685 bp SacI-XhoI fragment of cs44 with the SacI-XhoI-cut pSUN5 vector (Figure 8, construct map).

In Figure 8, the *AP3P* fragment includes the modified AP3P promoter (771 bp), the *P-sense* fragment includes the 312 bp promoter fragment of epsilon-cyclase in the sense orientation, the *intron* fragment includes the IV2 intron of the potato ST-LS1 gene, and the *P-anti* fragment includes the 312 bp promoter fragment of epsilon-cyclase in antisense orientation.

The expression vector pS5CI7 was prepared by ligating the 2445 bp SacI-XhoI fragment of cs45 with the SacI-XhoI-cut pSUN5 vector (Figure 9, construct map).

In Figure 9, the *CHRC* fragment includes the CHRC promoter (1537 bp), the *P-sense* fragment includes the 312 bp promoter fragment of epsilon-cyclase in the sense orientation, the *intron* fragment includes the IV2 intron of the potato ST-LS1 gene, and the *P-anti* fragment includes the 312 bp promoter fragment of epsilon-cyclase in antisense orientation.

The expression vector pS5CI7 was prepared by ligating the 3219 bp SacI-XhoI fragment of cs46 with the SacI-XhoI-cut pSUN5 vector (Figure 10, construct map).

In Figure 10, the *CHRC* fragment includes the CHRC promoter (1537 bp), the *P-sense* fragment includes the 312 bp promoter fragment of epsilon-cyclase in the sense orientation, the *intron* fragment includes the IV2 intron of the potato ST-LS1 gene, the *P-anti* fragment includes the 312 bp promoter fragment of epsilon-cyclase in antisense orientation, and the *AP3P* fragment includes the 771 bp AP3P promoter fragment in the antisense orientation.

Example 6: Preparation of transgenic Tagetes plants

Tagetes seeds are sterilized and placed on germination medium (MS medium; Murashige and Skoog, Physiol. Plant. 15(1962), 473-497) pH 5.8, 2% sucrose). Germination takes place in a temperature/light/time interval of 18 to 28°C/20 to 200 µE/3 to 16 weeks, but preferably at 21°C, 20 to 70 µE, for 4 to 8 weeks.

All the leaves of the plants which have developed in vitro by then are harvested and cut perpendicular to the mid rib. The leaf explants produced in this way with a size of 10 to 60 mm^2 are stored during the preparation in liquid MS medium at room temperature for a maximum of 2 h.

The Agrobacterium tumefaciens strain EHA105 was transformed with the binary plasmid PS5AI3. The transformed A. tumefaciens EHA105 strain was grown overnight under the following conditions: a single colony was inoculated in YEB (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose, 0.5% magnesium sulfate $\times$ 7 H_20) with 25 mg/l kanamycin and grown at 28°C for 16 to 20 h. The bacterial suspension was then harvested by centrifugation at

6000 g for 10 min and resuspended in liquid MS medium such that an OD_{600} of approx. 0.1 to 0.8 was produced. This suspension was used for the cocultivation with the leaf material.

Immediately before the cocultivation, the MS medium in which the leaves have been stored is replaced by the bacterial suspension. The leaves were incubated in the suspension of agrobacteria for 30 min while shaking gently at room temperature. The infected explants are placed on an MS medium with grown regulators such as, for example, 3 mg/l benzylaminopurine (BAP) and 1 mg/l indolyl acetic acid (IAA), which has been solidified with agar (e.g. 0.8% plant agar (Duchefa, NL)). The orientation of the leaves on the medium has no significance. The explants are cultivated for 1 to 8 days, but preferably for 6 days, during which the following conditions can be used: light intensity: 30 to 80 $\mu mol/m^2 \times s$, temperature: 22 to 24°C, 16/8 hours of light/dark alternation. The cocultivated explants are then transferred to fresh MS medium, preferably with the same growth regulators, this second medium additionally containing an antibiotic to suppress bacterial growth. Timentin in a concentration of from 200 to 500 mg/l is very suitable for this purpose. The second selective component employed is one for selecting for successful transformation. Phosphinothricin in a concentration of from 1 to 5 mg/l selects very efficiently, but other selective components are also conceivable according to the process to be used.

After one to three weeks in each case, the explants are transferred to fresh medium until plumules and small shoots develop, and these are then transferred to the same basal medium including Timentin and PPT or alternative components with growth regulators, namely, for example, 0.5 mg/l indolylbutyric acid (IBA) and 0.5 mg/l gibberilic acid GA_3, for rooting. Rooted shoots can be transferred to a glasshouse.

In addition to the method described, the following advantageous modifications are possible:

- before the explants are infected with bacteria, they can be preincubated on the medium described above for the cocultivation for 1 to 12 days, preferably 3 to 4. This is followed by infection, cocultivation and selective regeneration as described above.

- the pH for the regeneration (normally 5.8) can be lowered to pH 5.2. This improves control of the growth of agrobacteria.

- addition of $AgNO_3$ (3 to 10 mg/l) to the regeneration medium improves the condition of the culture, including the regeneration itself.

- components which reduce phenol formation and are known to the skilled worker, such as, for example, citric acid, ascorbic acid, PVP and many others, have beneficial effects on the culture.

- liquid culture medium can also be used for the whole process. The culture can also be incubated on commercially available supports which are positioned on the liquid medium.

According to the transformation method described above, the following lines were obtained using the following expression constructs:

CS30-1, CS30-3 and CS30-4 were obtained with pS5AI3.

Example 7: Characterization of the transgenic plants

The flower material of the transgenic Tagetes erecta plants of Example 6 was crushed in liquid nitrogen and the powder (about 250 to 500 mg) was extracted with 100% acetone (three times, 500 µl each). The solvent was evaporated and the carotenoids were resuspended in 100 µl of acetone.

Using a C30 reverse phase column it was possible to distinguish between the carotenoid mono- and diesters. The HPLC run conditions were virtually identical to a published method (Frazer et al. (2000), Plant Journal 24(4): 551-558). It was possible to identify the carotenoids on the basis of the UV-VIS spectra.

Table 1 depicts the carotenoid profile in Tagetes petals of the transgenic Tagetes plants prepared according to the examples described above and of control Tagetes plants. All of the carotenoid quantities are given in [µg/g] fresh weight, with percentages of change compared to the control plant being indicated in parentheses.

In comparison with the genetically unmodified control plant, the genetically modified plants have a distinctly increased content of carotenoids of the "β-carotene pathway", such as, for example, β-carotene and zeaxanthin, and a distinctly reduced content of carotenoids of the "α-carotene pathway", such as lutein, for example.

Table 1

Plant	Lutein	β–Carotene	Zeaxanthin	Violaxanthin	Total carotenoids
Control	260	4.8	2.7	36	304
CS 30–1	35 (–86%)	13 (+170%)	4.4 (+62%)	59 (+63%)	111 (–63%)
Control	456	6.4	6.9	58	527
CS 30–3	62 (–86%)	13 (+103%)	8.9 (+29%)	75 (+29%)	159 (–70%)
CS 30–4	68 (–85%)	9.1 (+42%)	5.7 (–17%)	61 (+5%)	144 (–73%)
Control	280	4.1	2.6	42	329
CS 32–9	69 (–75%)	5.5 (+34%)	2.3 (–12%)	25 (–38%)	102 (–69%)

Comparative example 1: Reduction of ε-cyclase activity in Tagetes erecta by antisense

Using conventional methods known to the skilled worker, a Tagetes erecta antisense line, CS32-9, in which the ε-cyclase activity was reduced by antisense was prepared as comparative example. The carotenoid profile of this line (CS32-9), measured by the method described above, is likewise depicted in Table 1.